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The effects of astrocytes on differentiation of neural stem cells are influenced by knock-down of the glutamate transporter, GLT-1

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ABSTRACT

The majority of glutamate released during neurotransmission is uptaken into astrocytes through the glial glutamate transporter GLT-1, by which extracellular glutamate is inactivated. In this study, we determined whether GLT-1 mediated the astrocyte regulation of the cell fate of neural stem/progenitor cells (NSCs) by glutamate reuptake. The astrocytes stimulated neuronal lineage selection but inhibited glial lineage cells. However, all these effects were reversed after siRNA-targeting GLT-1 was delivered into astrocytes by lentiviral vectors. NSC and astrocyte co-culture also increased the synaptophysin protein levels of NSC-derived new neurons through GLT-1. Glutamate was found to be present in the supernatants of the co-culture and astrocytes under different medium conditions, which may be attributed to the slower rate of clearance of the released glutamate. Dysfunctional glutamate reuptake may be the major consequence of GLT-1 functional silence in astrocytes. These results indicated that astrocytes regulated NSCs in reactive astrogliosis, neuronal generation, and synaptic function through GLT-1.

1. Introduction

Neurogenesis occurs in the two germinal zones of the adult mammalian central nervous system (CNS), including the subgranular zone of the hippocampus (Taupin and Gage, 2002). Neural stem/progenitor cells (NSCs) isolated from this region retain the potential of self-renewal and multi-lineage differentiation. These NSCs also generate new granular layer neurons in the dentate gyrus throughout a lifespan (Abrous et al., 2005). This property contributes to functional plasticity under both physiological and pathological conditions. For example, ischemia-stimulated compensatory neurogenesis can enable rehabilitation after injury and facilitate cognitive recovery (Raber et al., 2004). We have previously observed depressive behaviors in post-stroke animals in which ischemia-stimulated neurogenesis has been attenuated (Wang et al., 2008), which revealed the potential etiology of post-stroke depression.

In addition to the neurogenic capacity of NSCs, they can divide and differentiate into glial lineages (Palmer et al., 1997, 2000; Steiner et al., 2004). Astrocytes are fundamental to the onset, progression, and outcome of neuropathological processes. These cells limit damage and promote the revascularization of surrounding tissue through reactive astrogliosis (Giaume et al., 2007; Heneka et al., 2010; Nedergaard et al., 2010). However, the majority of NSCs differentiate into astrocytes instead of neurons in damaged lesions, thereby limiting the use of NSCs as potential therapeutic agents for brain injury. We have previously found that chronic unpredicted mild stress can alter ischemia-induced neurogenic fate by increasing the glial lineage differentiation of NSCs (Wang et al., 2008). Therefore, controlling the direction of NSC differentiation is a major challenge in treating CNS diseases (Emsley et al., 2005).

Faijerson et al. (2006) modeled astrogliosis *in vitro* using mechanical lesions of primary astrocytes. They found that lesioned astrocytes stimulate the astrocytic differentiation of NSCs without affecting neuronal or oligodendrocytic differentiation. Moreover, co-culturing NSCs with lesioned astrocytes reveals that several of these NSC-derived astrocytes participate in glial scar formation *in vitro*. Thus, astrocytes likely affect the cell behavior and fate of NSCs after brain injury. However, the molecular mechanisms underlying astrocyte-induced NSC activity especially during diseases remain elusive.

Astrocytes, which are components of the tripartite synapse, modulate neurotransmission and control the extracellular level of neurotransmitters (Araque et al., 1999; Danbolt, 2001; Grosche et al., 1999; Halassa and Haydon, 2010; Perea et al., 2009; Wilhelmsson et al., 2006). Therefore, astrocytes are essential to the glutamate–glutamine cycle, which is fundamental for the synaptic plasticity associated with cognitive processes (Kvamme, 1998; McKenna, 2007). Astrocytic glutamate uptake prevents glutamate excitotoxicity (Danbolt, 2001). However, the disturbance of

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astrocytic-based glutamate homeostasis may lead to neurotransmitter imbalance, neuronal malfunction, impaired cognition, and death (Choi, 1992; Walton and Dodd, 2007). The majority of glutamate released during neurotransmission is uptaken into astrocytes by the mediation of a family of high-affinity glial glutamate transporters including GLAST/EAAT1 and GLT-1/EAAT2, which is the only mechanism by which extracellular glutamate is inactivated. Several studies have documented that GLT-1 is critical to the maintenance of extracellular glutamate below neurotoxic levels (Kanai et al., 1997; Rothstein et al., 1996; Rothstein et al., 1994). Diminished expression of GLT-1 contributes to neurodegenerative disorders (Choi, 1988), including Alzheimer's disease (Li et al., 1997; Masliah et al., 2000).

Studies have indicated that dysfunctional glutamate reuptake promotes neuronal death after brain injury (Rao et al., 2000; Torp et al., 1995). However, the functional significance of glutamate transporter subtypes in inducing the cell fate of NSCs has not yet been reported. In the present work, a cell co-culture of astrocytes and NSC medium was produced using Transwell dishes. We determined whether GLT-1 modulated the astrocyte regulation of the cell fate, reactive astrogliosis, neuronal differentiation, and synaptic formation of NSCs by delivering the siRNA-targeting GLT-1 into astrocytes using lentiviral vectors.

2. Materials and methods

2.1. Preparation of a lentiviral vector expressing short hairpin RNA (shRNA) against GLT-1

Vectors were constructed using standard cloning procedures. Oligonucleotides encoding both strands of the targeting sequence were annealed and ligated into the MluI/ClaI site in pLVTHM vector. The nucleotide sequence of shRNAs targeting rat GLT-1 gene was as follows: Rat-GLT-1-sh: 5′-GAGCTACCTGTTTCGGAAT-3′. Their scrambled sequence used as control was 5′-TTCTCCGAACGTGTCACGT-3′. About 1 μL of annealed oligonucleotides, 2 pmol of MluI/ClaI digested vector pLVTHM, 1 μL of 10× ligation buffer, 1 μL of T4 DNA ligase (TaKaRa), and ddH2O were mixed together. About μL of the ligation mixture was incubated at 4 °C overnight. Approximately 5 μL of ligation products was transfected into competent *Escherichia coli* DH5α (TaKaRa). The vector including the shRNA was confirmed by sequencing (Supplementary Figure 1). About 10 μg of pLVTHM or pLVTHM/shRNA and 10 μg of packing mix pRsv-REV, pMDlg-pRRE, and pMD2.G (Invitrogen) were mixed together and then transfected into 293T cells according to the manual of Lipofectamine 2000 (Invitrogen). The transfected medium was replaced with fresh medium 6 h after transfection. Culture supernatants were collected 48 h after transfection and referred to as Lv-shGLT-1 or Lv-NC. The transfecting supernatants were purified by ultracentrifugation. Virus titers were calculated at 72 h after virus infection in 293T cells by counting the number of green fluorescent proteins expressing foci divided by the dilution factor.

2.2. Cell culture and transfection

Following the method of McManus and Trombetta (1995), rat hippocampal astrocytes from 1- to 3-day-old Sprague–Dawley rat pups were cultivated in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 nutrient media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1 mg/mL gentamycin. The astrocytes were purified using differential speed, shaken at room temperature, and then washed for 10 min with DMEM to remove microglia and oligodendrocytes. The astroglial lineage of the resulting cells was confirmed by the presence of glial fibrillary acidic protein (GFAP). Astrocytes were cultured at a density of 5×10^4 cells/mL in a culture bottle to synchronize for cell transfection. For cell infection, astrocytes were incubated with Lv-shGLT-1 or Lv-NC for 120 h, and the medium was replaced 24 h after adding the lentiviruses (MOI = 10). Stably transfected clones were selected using puromycin dihydrochloride (Sigma–Aldrich) selection.

The silenced gene and protein expression of GLT-1 were confirmed by real-time PCR and Western blot analysis. Total RNA was isolated from cultured cells using Trizol (Ambion). Total RNA (1 μg) was used as a template for RT with a High-Capacity cDNA RT Kit (Applied Biosystems). Real-time RT-PCR was performed by monitoring the increase in fluorescence of SYBR Green dye using the Rotor-Gene 3000 Real-time PCR apparatus (Corbett Research) according to the manufacturer's instructions. All PCR primers were designed by Primer Express Software V2.0 (Table 1). All measurements were conducted in triplicate. For immunoblot analysis, cells were collected and homogenized in ice-cold lysis buffer [containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L Na-pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 g/mL leupeptin, and 1 mmol/L PMSF]. The cells were then centrifuged at 16,000 rpm for 15 min. Protein samples were electrophoresed on 10% Bis–Tris gels (Invitrogen) and transferred onto polyvinylidene fluoride membranes according to the manufacturer's instructions (Invitrogen). Immunoblot analyses were carried out with the following antibodies: anti-GLT-1 (1:500, Santa Cruz) and anti-β-actin (1:2000, Santa Cruz). Protein expression level was quantified by densitometry (TotalLab, version 1.1, UK) for each blot. The density ratio for the bands was calculated. All data were expressed relative to control as follows: relative density (%) = density (study groups)/density (control group) × 100.

2.3. Preparation and co-culture of NSCs with astrocytes using Transwell dishes

NSCs were prepared as described by Wang et al. (2009) with minor modifications. In a typical procedure, an ordinal pregnant Sprague–Dawley rat was intraperitoneally injected with 0.4% sodium phenobarbital and decapitated at 13.5 gestational days. The brains were removed from the embryo and placed in DMEM/F12 (1:1) medium (Gibco) containing 20 mg/mL basic fibroblast growth factor (Gibco), 20 mg/mL epidermal growth factor (EGF, Gibco), 10 mg/mL N2 (Gibco), and 20 mg/mL B27 supplement (Gibco). Primary proliferative neurospheres were formed and passaged by mechanical dissociation. In a Transwell dish, NSCs were plated in poly-l-lysine pretreated six-well dishes (Nunc), and protoplasmic astrocytes were cultured in the insert (Millipore, MA, USA). The pore size of the Transwell dish was 0.1 μm. Monoclonal NSCs were subcultured at a density of 5×10^4 cells/mL and divided as follows: group A, NSCs without astrocytes; group B (independent of the sequence of the hairpin structure of the virus carrier; provided by Shanghai Sangon Biotech Co., Ltd), NSCs co-cultured with control vector infected astrocytes; and group C, NSCs co-cultured with lentiviral vector-based GLT-1 RNAi-transfected astrocytes. Co-cultured cells were maintained in NB + 2% B27 medium. Half of the medium was changed every 3 days. After 7 days of co-culture, we

Table 1
The primers for real-time PCR.

Gene	Primers
GLT-1-F	5′ CCTGTTTCGGAATGCCTTTGTGCT 3′
GLT-1-R	5′ TATACGGTCGGAGGGCAAATCCAA 3′
β-actin-F	5′ TTGCTGACAGGATGCAGAAGGAGA 3′
β-actin-R	5′ ACTCCTGCTTGCTGATCCACATCT 3′

Table 2
Expression of GLT-1 mRNA in groups, as determined by the $2^{-\Delta\Delta Ct}$ relative quantification algorithm.

Group	$Ct_{\beta\text{-actin}}$	$Ct_{GLT\text{-}1}$	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
Vector control (Control)	8.96 ± 3.51	19.61 ± 0.32	10.65 ± 0.52	0.05 ± 0.52	1.00 ± 0.36
Glut1 siRNA astrocytes (Sample)	9.10 ± 0.35	21.28 ± 0.24	12.18 ± 0.55	1.58 ± 0.54	0.36 ± 0.13[#]

Expression of GLT-1 mRNA analysed by the $2^{-\Delta\Delta Ct}$ relative quantification algorithm.
$\Delta Ct = Ct_{GLT\text{-}1} - Ct_{reference}$. $\Delta\Delta Ct = (Ct_{GLT\text{-}1} - Ct_{reference})_{control} - (Ct_{GLT\text{-}1} - Ct_{reference})_{sample}$.
The N-fold differential expression in a specific gene of the sample related to the ΔΔCt control counterpart was determined and expressed by $2^{-\Delta\Delta Ct}$. Values are means ± S.D. $N = 3$ for each group.
[#] Indicates $P < 0.01$, Glut1 siRNA astrocytes (Sample) vs. Vector control (Control).

observed the behavior and fate of the NSCs under proliferation or differentiation conditions. The abovementioned serum-free mitogenic medium was used for proliferation and differentiation studies. The NSCs that adhered onto glass coverslips precoated with poly-l-ornithine/laminin were induced to differentiate using DMEM/F12 (1:1) medium containing 10% FBS for 3 days to 7 days.

2.4. Evaluation of cell growth

Cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide absorbance and cell count assay. The absorbance at 570 nm was measured in solubilized cells using spectrophotometry (Hitachi). The cell growth rate was expressed as a percentage of values obtained in the vehicle control. In another set of experiments, primary spheres were collected, passaged by mechanically dissociating neurospheres in 0.2 mL of serum-free media, and then plated at 500 cells/well in uncoated 96-well plates. The number of spheres was counted after 3 and 7 days *in vitro*. Up to 6–8 wells per condition tested were counted.

2.5. Evaluation of differentiation and synaptic density

NSCs were Transwell co-cultured with astrocytes onto poly-l-ornithine/laminin-coated coverslips for 7 days. 5-Bromodeoxyuridine (BrdU) was dissolved in phosphate-buffered saline (PBS) and added to the culture medium at a final concentration of 10 μM for 15 min at 37 °C. The NSCs were washed twice and further incubated at 37 °C for 10 h, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 30 min, and washed. The fixed cells were incubated with anti-microtubule-associated protein 2 (MAP2; 1:500, Invitrogen) followed by incubation with FITC-conjugated anti-rabbit IgG antibody (1:1000, Vector) or anti- BrdU antibody (1:200, Chemicon) followed by rhodamine-conjugated goat anti-mouse immunoglobulin (IgG) antibody (Vector, 1:500 dilution). Nuclear staining was performed by incubation with FITC-conjugated anti-rabbit IgG antibody (1:1000, Vector), and NSCs were observed using a confocal laser scanning microscope (Zeiss LSM510, Carl Zeiss) equipped with a 40× objective at excitation/emission wavelengths of 535/565 nm (Rhodamine, red) and 470/505 nm (FITC, green).

The examine was repeated three times, and 8–10 coverslips (each group) were examined each time, approximately 100–200 cells were randomly counted per coverslip in a blind manner. Neuronal and glial differentiations were expressed as the percentages of $MAP2^+$ and $GFAP^+$ cells quantified by normalizing total MAP2 or GFAP cells to the total number of cells labeled with BrdU (approximately 100–200 cells were randomly counted per coverslip in a blind manner; $n = 3$ dissections).

The percentage of synaptophysin positive cells was expressed as a percentage of the number of $synaptophysin^+/MAP2^+$ cells. The fixed cells were incubated with anti-MAP2 (1:500) and anti-synaptophysin antibodies (1:500, Abcam) overnight at 4 °C, visualized with rhodamine-conjugated goat anti-mouse IgG antibody (1:1000, Vector) and FITC-conjugated anti-rabbit IgG antibody (1:500, Vector) for 1 h at room temperature. The cells were then counterstained with 300 nM 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) for 3 min. The labeled cells were scanned using a confocal laser scanning microscope.

The synaptophysin protein levels were investigated using Western immunoblot analysis. Cells were collected and homogenized in ice-cold lysis buffer (containing 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L Na-Pyrophosphate, 1 mmol/L b-glycerophosphate, 1 mmol/L Na3VO4, 1 g/mL leupeptin, and 1 mmol/L PMSF) and then centrifuged at 16,000 rpm for 15 min. Protein samples were electrophoresed on 10% Bis–Tris gels (Invitrogen) and transferred onto polyvinylidene fluoride membranes according to the manufacturer's instructions (Invitrogen). Immunoblot analyses were carried out with the following antibodies: anti-synaptophysin (1:500, Abcam) and anti-β-actin (1:2000, Santa Cruz). Protein expression level was quantified by densitometry (TotalLab, version 1.1, UK) for each blot. The density ratio for the bands was calculated. All data were expressed relative to the control as follows:

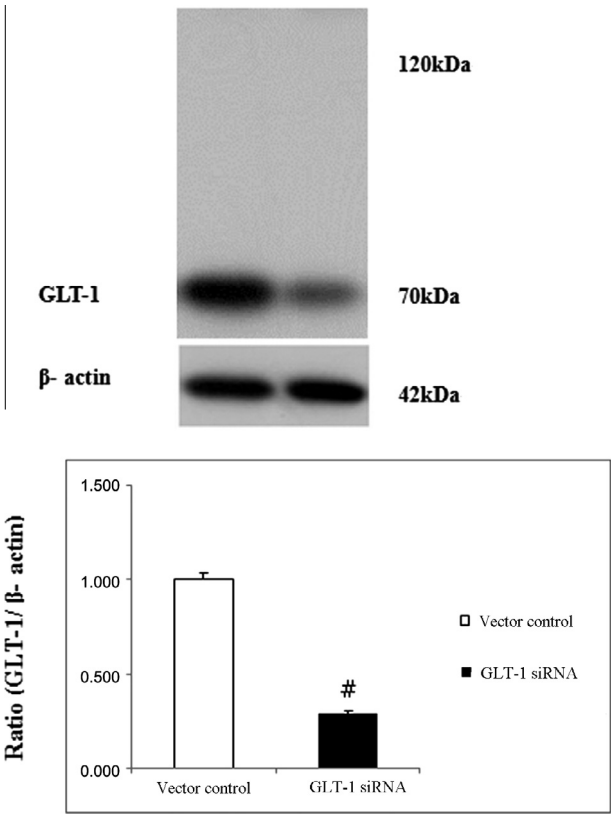

Fig. 1. Knock-down efficiency of the shRNAs. Immunoblot analysis indicates that shRNAs delivered by lentiviral vectors silenced GLT-1 expression in astrocytes. #indicates $P < 0.01$, GLT-1 siRNA astrocytes vs. vector control.

relative density (%) = density (study groups)/density (control group) × 100.

2.6. Glutamate level and astrocyte expression of supernatant

Glutamate secretion was measured by a mouse anti-rat glutamate enzyme-linked immunosorbent assay (Abcam). The supernatant solution from the aforementioned proliferation and differentiation medium conditions was used as a sample 1, 3, and 7 days after co-culture. The optical density was measured at 450 nm on a standard micro-plate reader (BioRad). Three independent experiments were conducted.

3. Statistical analysis

Data were analyzed using Graph Pad Prism version 4.0. Values are presented as the mean ± SD. Student's *t*-test or Bonferroni correction was performed for statistical evaluation. A probability value of $P < 0.05$ was considered statistically significant.

4. Results

4.1. Reduced efficacy of shRNAs

shRNAs delivered into astrocytes by lentiviral vectors were used to functionally silence GLT-1 expression, as confirmed by real-time PCR and Western blot analyses. Real-time PCR analysis indicated that the shRNA delivered by lentiviral vectors functionally reduced astrocyte GLT-1 expression to 35.5% of the control levels (Table 2). Immunoblot analysis indicated that the protein level of GLT-1 decreased by 71.5% when astrocytes were infected with GLT-1 shRNA virus (Fig. 1).

4.2. Effect of GLT-1 on the proliferation of NSCs upregulated by astrocytes

Astrocytes stimulated NSC proliferation after 3 and 7 days of co-culture (149.0% ± 3.7% and 167.3% ± 4.0% vs. 100%, $n = 3$, all $P < 0.001$), respectively. Cell growth exhibited no significant decrease after NSCs were co-incubated with GLT-1-RNA-silenced astrocytes (145.3% ± 2.5% vs. 149.0% ± 3.7%, 162.8% ± 3.5% vs. 167.3% ± 4.0%, $n = 3$, all $P > 0.05$; Figs. 2A and B). Morphologically, the astrocytes showed increased self-renewal capacity, proliferating (12 ± 2/500 vs. 6 ± 1/500, 19 ± 2/500 vs. 8 ± 1/500) after 3 and 7 days *in vitro* ($n = 3$, all $P < 0.001$). The number of spheres exhibited no significant decrease after incubating GLT1-silenced astrocytes with NSCs compared with those observed with NSCs co-cultured with astrocytes (11 ± 2/500 vs. 12 ± 2/500, 18 ± 1/500 vs. 19 ± 2/500, $n = 3$, all $P > 0.05$; Fig. 2C and D).

4.3. Effect of GLT-1 on the differentiation of NSCs and synaptophysin level upregulated by astrocytes

Resultant cultures were analyzed for neuronal and astrocytic numbers by immunohistochemical staining with MAP-2 Ab/BrdU Ab and GFAP Ab/BrdU Ab (Figs. 3A and 4A). As shown in Figs. 3B and 4B, immunocytochemistry analysis revealed that astrocytes significantly increased the percentage of the numbers of MAP2 and BrdU-immunoreactive cells with neuronal morphology (22.3% ± 1.2% vs. 15.7% ± 0.6%, $n = 3$, $P < 0.001$) and decreased the

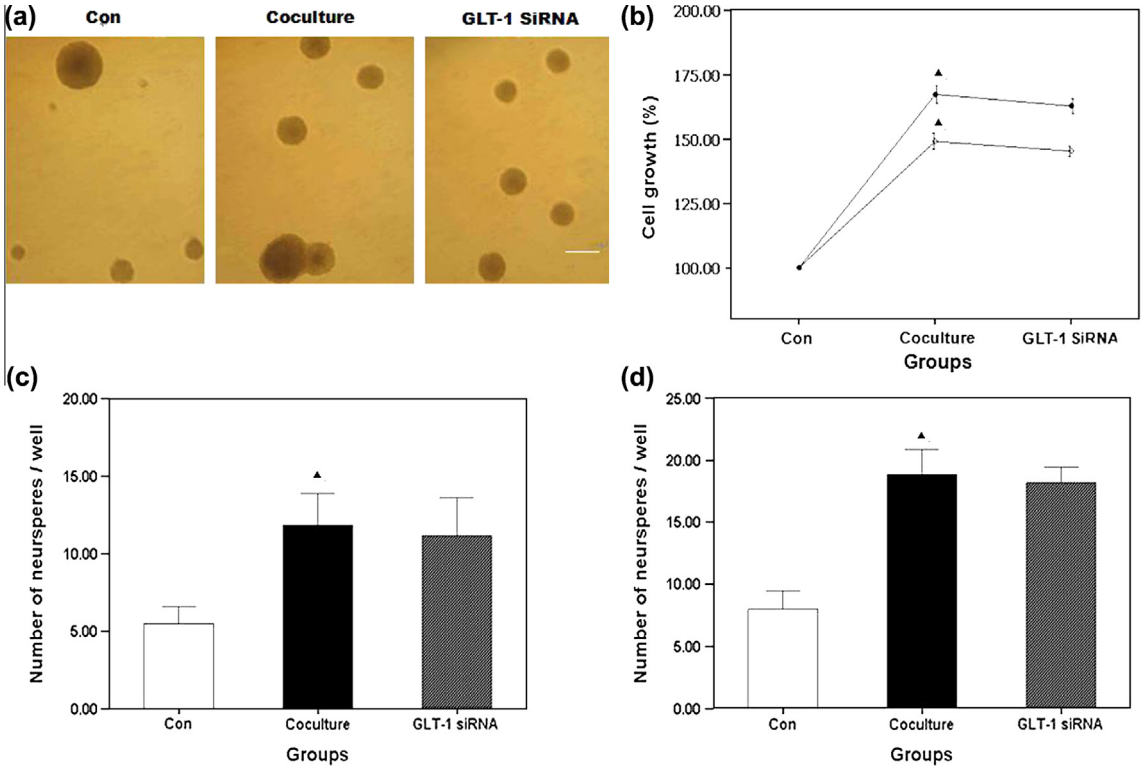

Fig. 2. Effect of GLT-1 on the proliferation of NSCs upregulated by astrocytes. (A) Effect of GLT-1 interference astrocytes on morphological changes of NSCs. NSCs were prepared from ordinal SD rat embryo brains. GLT-1 expression level was downregulated by loss-of-function approaches using RNA interference lentiviral gene transfer in astrocytes. Bar represents 100 μm. (B) Effect of GLT-1 interference astrocytes on the cell growth rate of NSCs (open circle, 3-day; closed circle, 7-day co-culture). Statistically significant differences between NSCs coculture with control vector infected astrocytes (Coculture) and NSCs only (Con), as determined by the Bonferroni correction, are indicated (▲$P < 0.001$). (C) Effect of GLT-1 interference astrocytes on the number of neurpheres (3-day co-culture). Statistically significant differences between NSCs coculture with control vector infected astrocytes (Coculture) and NSCs only (Con), as determined by the Bonferroni correction, are indicated (▲$P < 0.001$). (D) Effect of GLT-1 interference astrocytes on the number of neurpheres (7-day co-culture). Statistically significant differences between NSCs coculture with control vector infected astrocytes (Coculture) and NSCs only (Con), as determined by the Bonferroni correction, are indicated (▲$P < 0.001$).

percentage of the numbers of GFAP and BrdU-immunoreactive cells with astrocytic morphology (57.7% ± 2.1% vs. 69.7% ± 1.5%, $n = 3$, $P < 0.001$). However, all these values were reversed after GLT-1-silenced astrocytes were co-cultured with NSCs (16.7% ± 0.6% vs. 22.3% ± 1.2%, 70.7% ± 2.5% vs. 57.7% ± 2.1%, $n = 3$, all $P < 0.01$). The decreased number of new neurons significantly increased glial differentiation. Immunocytochemistry and immunoblot analysis revealed that GLT-1 siRNA downregulated the astrocyte-induced elevation of the mean percentage of synaptophysin-expressing neurons (22.3% ± 2.5% vs. 39.7% ± 3.2%, $n = 3$, $P < 0.01$) (Fig. 5A and B) and synaptophysin protein expression (Fig. 5C) after co-culturing with NSCs.

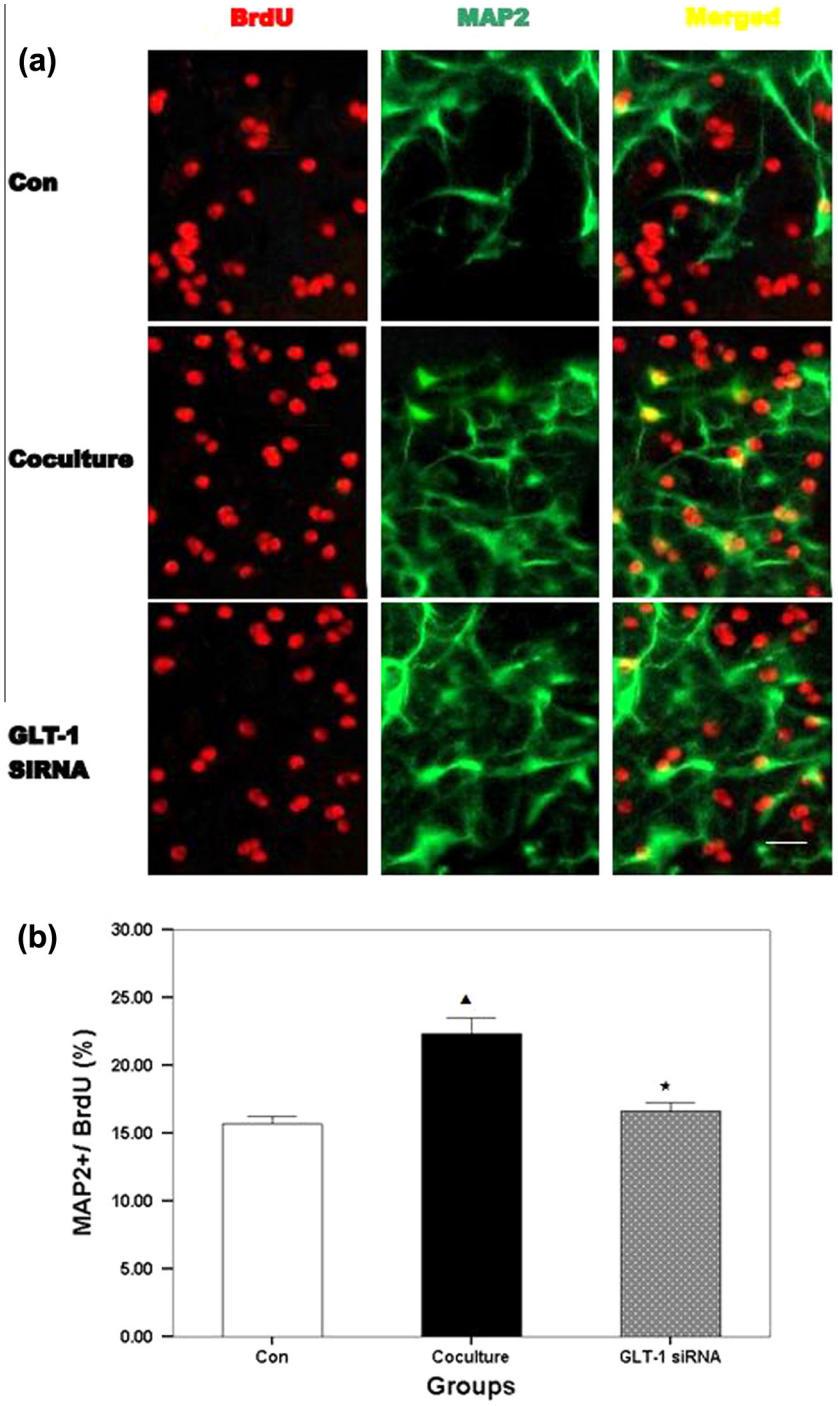

Fig. 3. GLT-1 mediated astrocytes-upregulated the neuronal differentiation of NSCs. (A) Confocal microscopy of representative double-stained images of differentiated neurosphere immunostained with anti-BrdU Ab (Left, red) and anti-MAP2 Ab (Centre, green). BrdU and MAP2 labelling are shown in both isolated and overlapping cases (Right). The experiment was repeated thrice, and similar results were obtained each time. Bar represents 25 μm. NSCs were prepared from ordinal SD rat embryo brains. GLT-1 expression level was downregulated by loss-of-function approaches using RNA interference lentiviral gene transfer in astrocytes. (B) Effect of GLT-1 interference astrocytes on the neuronal differentiation of NSCs (7-day co-culture). Statistically significant differences between NSCs coculture with control vector infected astrocytes and NSCs only (Coculture vs. Con), as determined by the Bonferroni correction, are indicated (▲$P < 0.001$). Statistically significant differences between NSCs coculture with GLT-1 interference astrocytes and NSCs coculture with control vector infected astrocytes (GLT-1 siRNA vs. Coculture), as determined by the Bonferroni correction, are indicated (★$P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

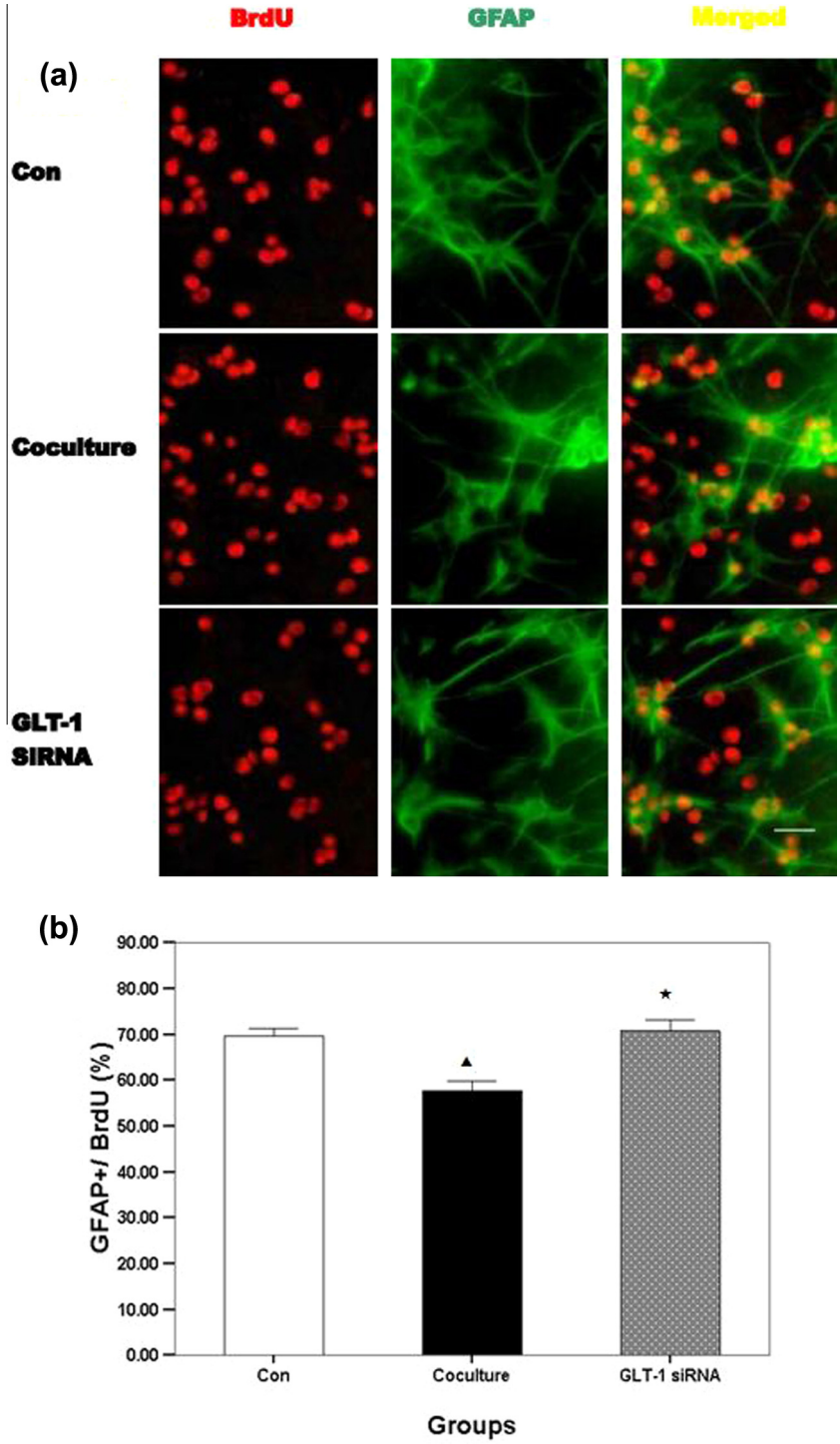

Fig. 4. GLT-1 mediated astrocytes-downregulated the glial differentiation of NSCs. (A) Confocal microscopy of representative double-stained images of differentiated neurosphere immunostained with anti-BrdU Ab (Left, red) and anti-GFAP Ab (Centre, green). BrdU and GFAP labelling are shown in both isolated and overlapping cases (Right). The experiment was repeated thrice, and similar results were obtained each time. Bar represents 50 μm. NSCs were prepared from ordinal SD rat embryo brains. GLT-1 expression level was downregulated by loss-of-function approaches using RNA interference lentiviral gene transfer in astrocytes. (B) Effect of GLT-1 interference astrocytes on the glial differentiation of NSCs (7-day co-culture). Statistically significant differences between NSCs coculture with control vector infected astrocytes and NSCs only (Coculture vs. Con), as determined by the Bonferroni correction, are indicated (▲$P < 0.001$). Statistically significant differences between NSCs coculture with GLT-1 interference astrocytes and NSCs coculture with control vector infected astrocytes (GLT-1 siRNA vs. Coculture), as determined by the Bonferroni correction, are indicated (★$P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.4. Content of glutamate in the supernatant and astrocytes under different medium conditions

Throughout a week of using the medium for proliferation study, the supernatant concentration of glutamate significantly increased when NSCs were co-cultured with both astrocytes and GLT-1 siRNA astrocytes. The supernatant level of glutamate increased by approximately by 30% after co-culturing NSCs with GLT-1 siRNA astrocytes for 3 and 7 days, and a statistically significant difference was observed (9.2 ± 1.0 nmol/L vs.

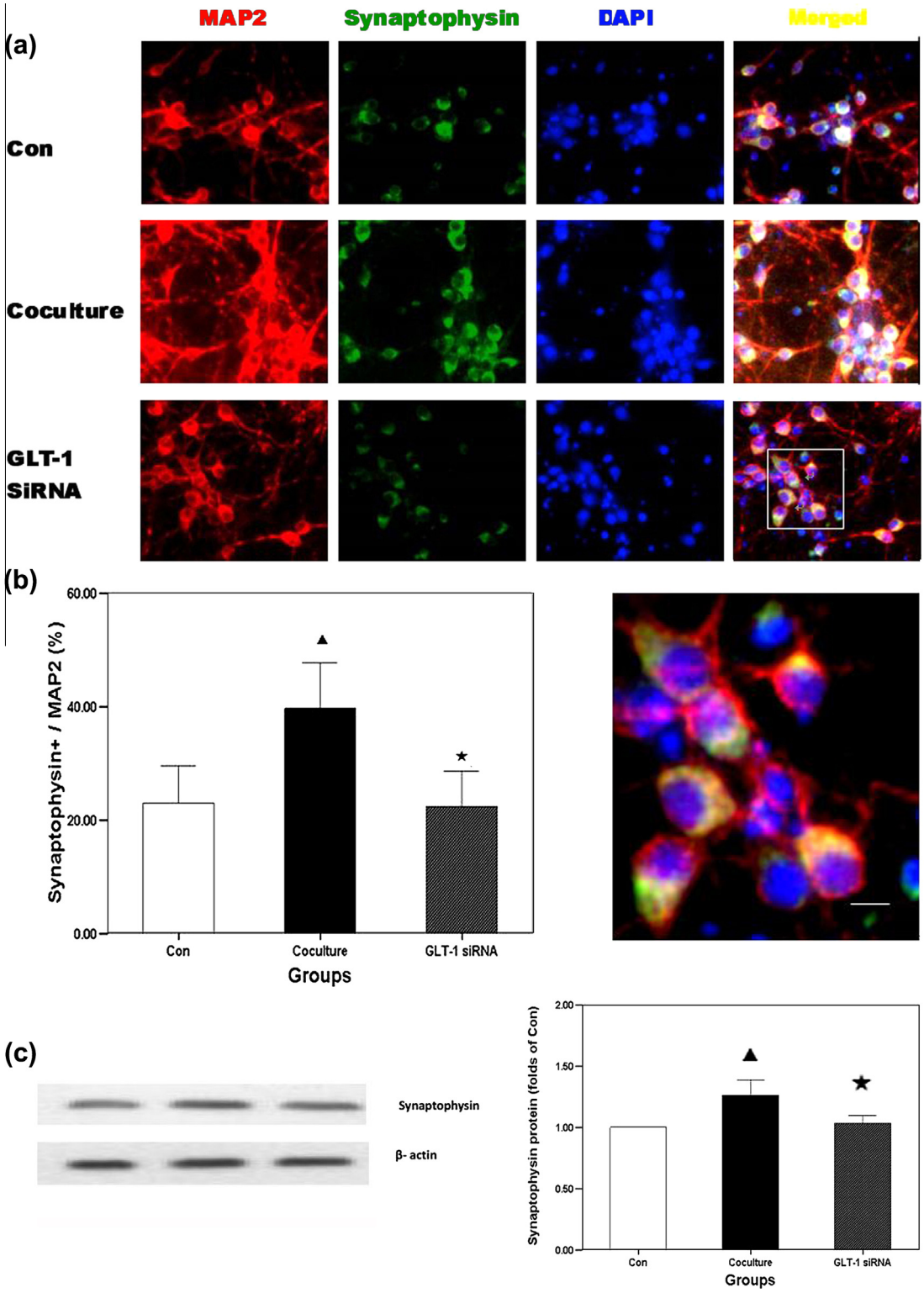

Fig. 5. GLT-1 mediated astrocytes-upregulated the synapse-related gene synaptophysin. (A) Confocal microscopy of representative three-stained images of new neurons immunostained with anti-MAP2 Ab (Red), anti- synaptophysin Ab (Green) and DAPI (Blue) when co-cultured with astrocytes on differentiation condition. Area inside white box is enlarged below. Labelling were shown in both isolated and overlapping cases. Bar represents 50 μm and 200 μm (partial larger image). (B) Synaptophysin positive new neurons when NSCs co-cultured with GLT1 interference astrocytes. (C) Western blotting analysis using anti-synaptophysin was performed to investigate protein levels of synaptophysin Representative photographs from three to four independent experiments are shown. NSCs were prepared from ordinal SD rat embryo brains. GLT-1 expression level was downregulated by loss-of-function approaches using RNA interference lentiviral gene transfer in astrocytes. Statistically significant differences between NSCs coculture with control vector infected astrocytes and NSCs only (Coculture vs. Con) are indicated ($^{\blacktriangle}P < 0.001$), or between NSCs coculture with GLT-1 interference astrocytes and NSCs coculture with control vector infected astrocytes coculture with NSCs (GLT-1 siRNA vs. Coculture) are indicated ($^{\bigstar}P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
The content of glutamate in the supernatant of different media conditions.

	1 day	3 day	7 day
Proliferation media			
Control (NSCs only)	4.2 ± 0.6	5.2 ± 0.6	5.5 ± 0.8
NSCs cocultured with astrocytes	6.4 ± 0.7#	7.1 ± 0.6#	8.0 ± 0.8#
NSCs cocultured with Glut1 siRNA astrocytes	7.4 ± 0.7	9.2 ± 1.0▲	10.4 ± 0.9▲
Differentiation media			
Control (NSCs only)	4.6 ± 0.6	7.6 ± 1.0	10.6 ± 0.8
NSCs cocultured with astrocytes	5.7 ± 0.5	8.6 ± 0.9	12.8 ± 1.3
NSCs cocultured with Glut1 siRNA astrocytes	6.5 ± 0.9	20.4 ± 1.7▲▲	30.7 ± 3.5▲▲

Units = nmol/L for all data in the table. $N = 10$ for each group.
# Indicates $P < 0.05$ NSCs cocultured with astrocytes compared to Control.
▲ Indicates $P < 0.05$.
▲▲ Indicates $P < 0.001$ NSCs cocultured with Glut1 siRNA astrocytes compared to NSCs cocultured with astrocytes.

7.1 ± 0.6 nmol/L; 10.4 ± 0.9 nmol/L vs. 8.0 ± 0.8 nmol/L, $n = 10$, all $P < 0.05$).

In the differentiation culture medium, the astrocytes slightly increased the supernatant concentration of glutamate after co-culturing with NSCs, but the difference was not significant. The level further increased to 6.5 ± 0.9, 20.4 ± 1.7, and 30.7 ± 3.5 nmol/L after co-culturing NSCs with GLT-1 siRNA astrocytes. A statistically significant difference was found 3 and 7 days after culture ($n = 10$, all $P < 0.001$), and GLT-1 siRNA astrocytes increased the supernatant level of glutamate by approximately 150% after co-culturing with NSCs at these two timepoints (Table 3).

5. Discussion

Studies have indicated that dysfunctional glutamate reuptake promotes neuronal death after brain injury. Although the regulation of NSCs has been extensively studied, studies on the proliferation and differentiation of NSCs after injury are limited. Reactive astrogliosis is a significant process that responds to almost all injuries and pathologies of the CNS. In these cases, reactive astrocytes are present and secrete numerous molecules and growth factors. In this work, we determined whether GLT-1 mediated synaptic function and the astrocyte regulation of the cell fate of NSCs by affecting glutamate-reuptake function. siRNAs targeting GLT-1 were delivered to astrocytes by lentiviral vectors. Notably, primary astrocytes can dramatically affect the cell fate of NSCs by stimulating neuronal lineage selection but potentially inhibit glial lineage cells through GLT-1. The cell growth of NSCs was not affected by astrocytes. Furthermore, co-cultures of NSCs and primary astrocytes increased the synaptic function of new NSC-derived neurons through GLT-1. Glutamate was detected in the supernatants of co-culture and astrocytes under different medium conditions, which can be attributed to the slower rate of clearance of the released glutamate. Such dysfunctional glutamate reuptake may be a major consequence of GLT-1 functional silence in astrocytes.

The results of this study supported the previous finding that hippocampal astrocytes promote the neuronal lineage selection of NSCs (Faijerson et al., 2006). However, inhibition of NSC-derived astrocytic differentiation was observed after NSCs were co-cultured with hippocampal astrocytes. This discrepancy may have resulted from the lesioned astrocytes and the conditioned medium from lesioned astrocytes used in the previous study, which reportedly promotes astrocytic differentiation. In the current proliferation study, astrocytes did not affect the proliferation of NSCs after co-culturing with NSCs. These results suggested that the effects on astrocytic differentiation primarily resulted from the increased induction of fate commitment to the neuronal lineage from NSCs. The pronounced effect on astrocytic differentiation observed in NSC co-cultured astrocytes indicated that this effect was primarily mediated by substances secreted from the astrocytes. Whether this effect also resulted from the basal reactive state inherent in primary astrocytes and the exact mechanism linking it to astrogliosis after brain injury are unclear. Additionally, synapses and synaptic integrity allow neurons to form circuits within the CNS. To characterize changes in the function of synapses, we measured the levels of synaptophysin, a glycoprotein component of synaptic vesicles and a synaptic marker. Synaptophysin reportedly regulates activity-dependent synapse formation (Tarsa and Goda, 2002). We observed that the presence of astrocytes profoundly increased the synaptophysin levels, and this effect was blocked after siRNA-targeting GLT-1 was delivered to astrocytes by lentiviral vectors. However, synaptophysin staining was distributed mainly in cell bodies rather than in the synaptic terminals, there are no more synapses to export it into, these findings may be an indication that GLT-1 in astrocytes was involved in the levels of synaptophysin but not change the synapse numbers.

Glutamate is critical to normal brain function and brain development. Perturbations of glutamate neurotransmission have severe consequences. Continuous glutamate release leads to a spreading of the process (Choi, 1992) termed excitotoxicity. Glutamate excitotoxicity has been implicated in a number of brain disorders, including epilepsy, amyotropic lateral sclerosis, Huntington's disease, Alzheimer's disease, ischemia, and trauma (Eid et al., 2004; Fujikawa, 2005; Hynd et al., 2004; Rego and de Almeida, 2005; Rothstein, 1996; Tannenberg et al., 2004). The capacity of glutamate to be highly toxic yet necessary for neurotransmission sets a fine balance between plasticity and pathology.

Previous studies have revealed that the glutamate–glutamine cycle is crucial to synaptic plasticity associated with cognitive processes. Thus, this cycle may be involved in the regulation of inhibitory synaptic strength, which can influence circuit excitability under normal and pathological conditions (Eid et al., 2004; Rae et al., 2003). Astrocytes are essential to glutamatergic transmission, being key elements for the synthesis of glutamate and for the glutamate–glutamine cycle. Meanwhile, one of the important sources of glutamate is the astrocytic glutamate–glutamine cycle, by which glutamate is uptaken by astrocytes through the astrocyte-specific GLT-1 and converted into glutamine by glutamine synthetase (Arriza et al., 1994; Pines et al., 1992). Glutamate must be rapidly and efficiently cleared from the synaptic cleft to reduce background signal traffic and prevent over-stimulation of receptors. This action is attributed to GLT-1, which is presumed by immunohistochemical and in situ studies to be astrocytic proteins. This phenomenon is responsible for removing extracellular glutamate in the CNS and is critical to the prevention of excitotoxicity (Choi et al., 1987) and modulation of synaptic transmission (Huang and Bergles, 2004).

This study provided a plausible mechanism underlying the effect of astrocytes on the cell fate of NSCs following brain injury. Our previous study has shown that chronic unpredicted mild stress alters ischemia-induced neurogenic fate by increasing the differentiation of NSCs to glial lineage cells. We observed that the stressors affecting the ischemia-related neurogenesis of the hippocampus are probably mediated by GLT-1. Both the increased release (caused by ischemia) and decreased reuptake caused by GLT-1 dysfunction may lead to increased duration of glutamate action in the synaptic cleft, thereby exacerbating ischemic neuronal damage and impairing neurogenesis. Reactive astrogliosis is regarded as dysfunctional glutamate reuptake because of the presence of GLT-1 after brain injury. Astrocytes seem to rapidly and efficiently clear glutamate from the synaptic cleft, thereby inhibiting excitotoxicity.

Interpretation of the data in this study has certain limitations. First, multipotent NSCs can differentiate into neurons, astrocytes, and oligodendrocytes under certain culture conditions (Emsley et al., 2005). Thus, under the differentiating medium condition, the effects of oligodendrocytes cannot be excluded. Astrocytes in culture are generally believed to express little or no GLT-1, and astrocyte activity is known to be mainly mediated by GLAST. At present, shRNAs delivered by lentiviral vectors do not fully silence GLT-1 expression in astrocytes. Further *in vivo* studies may be required to investigate the effects of astrocytes on neurogenesis and to provide more details on the mechanism involved. Despite these limitations, the findings raise the possibility that primary astrocytes can dramatically affect the cell fate of NSCs by stimulating neuronal lineage selection, inhibiting glial lineage cells, and potentially promoting synaptic function through GLT-1. Reactive astrogliosis is regarded as the cellular response to brain injury, such as stress after ischemia. Further investigations are required to determine the precise mechanism.

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